

Involvement of the Membrane Distal Catalytic Domain in Pervanadate-Induced Tyrosine Phosphorylation of Receptor Protein–Tyrosine Phosphatase α

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Received November 12, 1999

Receptor protein-tyrosine phosphatase α , RPTP α , is a typical transmembrane protein-tyrosine phosphatase (PTP) with two cytoplasmic catalytic domains. RPTP α became strongly phosphorylated on tyrosine upon treatment of cells with the PTP inhibitor pervanadate. Surprisingly, mutation of the catalytic site Cys in the membrane distal PTP domain (D2), but not of the membrane proximal PTP domain (D1) that harbors the majority of the PTP activity, almost completely abolished pervanadate-induced tyrosine phosphorylation. Pervanadate-induced RPTP α tyrosine phosphorylation was not restricted to Tyr789, a known phosphorylation site. Cotransfection of wild-type RPTP α did not potentiate tyrosine phosphorylation of inactive RPTP α -C433S, suggesting that RPTP α -mediated activation of kinase(s) does not underlie the observed effects. Mapping experiments indicated that pervanadate-induced tyrosine phosphorylation sites localized predominantly, but not exclusively, to the C-terminus. Our results demonstrate that RPTP α -D2 played a role in pervanadate-induced tyrosine phosphorylation of RPTP α , which may suggest that RPTP α -D2 is involved in protein-protein interactions. © 2000 Academic Press

Key Words: protein-tyrosine phosphatase; RPTP α ; D2; tyrosine phosphorylation.

Many important eukaryotic cell signaling pathways are controlled by protein tyrosine phosphorylation. The level of phosphotyrosine (P.Tyr) in cellular proteins is regulated by the balance of protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs). PTPs catalyze the hydrolysis of phosphoryl groups on Tyr residues in proteins. PTP family members share a conserved PTP domain of approximately 240 amino acids characterized by the signature motif [I/V]HCxAGxxR[S/T]G (1). Comparison of the crystal

structures of PTPs that have been solved thus far indicate that the tertiary structure of PTP domains is highly conserved (2–8).

The PTP family can be subdivided based on structural differences into receptor-like (RPTP) and cytosolic proteins (9). RPTPs, with CD45 as the founding member (10), consist of an extracellular domain, a single membrane spanning domain, and a cytoplasmic domain. Most RPTPs contain two tandemly repeated PTP domains in their cytoplasmic domain. For all RPTPs with two PTP domains, the majority of the catalytic activity resides within the membrane proximal PTP domain (D1) whereas the membrane distal domain, D2, displays little or no catalytic activity. The strongest indication for catalytic activity in D2 comes from work on RPTP α . RPTP α -D2 has been shown to have intrinsic activity when expressed in the absence of D1 (11, 12). Using artificial substrates, RPTP α -D2 was found to be only 10-fold less active than RPTP α -D1. However, the activity of RPTP α -D2 towards P.Tyr-containing peptides was shown to be several orders of magnitude lower than D1 (12). Inactivation of RPTP α -D1 is sufficient to abolish its biological activity (13). In addition, mutation of the critical Cys in CD45-D1, but not in D2, prevented restoration of CD45-mediated T cell signaling events, indicating that PTP activity in D1, but not D2, was essential for this function (14).

To elucidate the function of PTPs *in vivo* it is essential to know their natural substrates. Therefore, “substrate trapping” mutants have been designed. Mutation of the catalytic site Cys to Ala in YopH, a Yersinia PTP, resulted in a mutant that could still bind to substrates, but was no longer able to dephosphorylate them making this an excellent tool for identifying potential substrates (15). Other PTPs with catalytic site Cys to Ser or Ala mutations have been used successfully in substrate trapping experiments as well (16–19).

We set out to use substrate trapping mutants to identify potential substrates of RPTP α . To induce ty-

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rosine phosphorylation of potential substrates, RPTP α expressing cells were treated with the PTP inhibitor pervanadate to increase overall P.Tyr content of all proteins in the cell. No potential substrates were co-immunoprecipitated with a substrate trapping mutant of RPTP α . However, we observed a strong pervanadate-induced tyrosine phosphorylation of RPTP α itself. Surprisingly, mutation of the catalytic site Cys in RPTP α -D2 almost completely abolished pervanadate-induced tyrosine phosphorylation, while mutation of the catalytic site Cys in D1 had only moderate effects, suggesting that RPTP α -D2 and not D1 is somehow involved in RPTP α hyperphosphorylation. RPTP α is phosphorylated on Tyr789 *in vivo* (20). Here, we demonstrate that pervanadate-induced tyrosine phosphorylation was not restricted to Tyr789. Since pervanadate treatment of cells by itself was sufficient to induce tyrosine phosphorylation of RPTP α , we conclude that the PTK responsible is constitutively active. Site-directed mutagenesis and partial digestion of hyperphosphorylated RPTP α suggested that pervanadate mainly, but not exclusively induced tyrosine phosphorylation in the C-terminus. From these results, we conclude that RPTP α -D2 is involved in pervanadate-induced tyrosine phosphorylation of RPTP α , which may be mediated by protein-protein interactions via its catalytic site.

EXPERIMENTAL PROCEDURES

Cells and transfections. SK-N-MC neuroepitheloma cells were cultured in a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 (DF) medium supplemented with 10% fetal calf serum (Gibco, Paisley, UK). Transient transfection of SK-N-MC was done using calcium phosphate precipitation, as described (13).

Plasmids and site-directed mutagenesis. SV40 driven expression vectors, based on pSG-5 (21), for expression of RPTP α or mutants have been described (20). A hemagglutinin epitope tag was cloned at the N-terminal site of RPTP α as described (22). Site-directed mutagenesis was done on pSG-RPTP α or on pSG-HA-RPTP α . Mutations were verified by sequencing. The oligonucleotides that were used for site-directed mutagenesis were (numbering of residues in RPTP α according to (23)): RPTP α -C433S, ACC TGC ACT GGA GTG GAC CAC; RPTP α -C723S, CCC GGC ACT GGA GTG GTG CAC AGT; and RPTP α -Y789F, GCC TTT TCA GAT TTT GCC AAC TTC AAG.

Pervanadate treatment, immunoprecipitation and immunoblotting. Nearly confluent cells were treated with 1 mM orthovanadate and 1 mM H₂O₂, directly into the medium, generating pervanadate. The cells were treated for 30 min and lysed in CLB (50 mM HEPES pH 7.4, 150 mM NaCl, 1 mM MgCl₂, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 10 U/ml aprotinin, 1 μ M PMSF, 200 μ M sodium orthovanadate). Immunoprecipitation was done by incubation with anti-hemagglutinin epitope tag antibody (MAb 12CA5) and protein A-Sepharose (Pharmacia, Uppsala, Sweden) for 3 h at 4°C. The beads were washed four times with HNTG (20 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 10 U/ml aprotinin, 1 μ M PMSF), resuspended in Laemmli sample buffer and boiled for 5 min, and the samples were loaded onto 10% SDS-polyacrylamide gels. For immunoblotting analysis the material on the polyacrylamide gels was transferred to Immobilon (Millipore, Bedford, MA). Blots were probed with anti-P.Tyr antibody PY-20 (Transduction Laboratories, Lexington, KY), 12CA5 antibody, affinity-purified anti-RPTP α anti-

serum 5478, raised against the entire cytoplasmic domain, or affinity-purified anti-RPTP α antiserum 5476, raised against a C-terminal peptide (CYKVVQEYIDAFSDYANFK). The primary antibodies were detected with horseradish peroxidase (HRP)-conjugated anti-mouse antibody or HRP-conjugated protein A (Transduction Laboratories, Lexington, KY) and enhanced chemiluminescence (ECL). Before reprobing with different primary antibodies, the blots were stripped by incubation in stripping buffer (62.5 mM Tris, pH 6.8, 2% SDS, 100 mM β -mercaptoethanol) for 45 min at 70°C.

Proteolytic analysis. SK-N-MC cells, transiently transfected with wild type pSG-HA-RPTP α or pSG-HA-RPTP α -Y789F, were incubated with 1 mM orthovanadate (Sigma, St Louis, MO), and 1 mM H₂O₂. The cells were washed two times with PBS and lysed in CLB. Immunoprecipitation with anti-hemagglutinin epitope tag antibody (MAb 12CA5) was done as described above. The samples were loaded onto SDS-polyacrylamide gels. RPTP α was cut out and eluted from the gel, precipitated with trichloroacetic acid, and resuspended in V8 digestion buffer (125 mM Tris, pH 6.8, 1 mM EDTA, 0.1% SDS). The isolated RPTP α protein was digested with limited amounts of *Staphylococcus aureus* strain V8 protease (Sigma, St Louis, MO) at 37°C for 30 min. Following digestion, the samples were mixed with an equal volume of 2 \times Laemmli sample buffer, boiled for 5 min and loaded onto a 20% SDS-polyacrylamide gel. After electrophoresis, the gels were immunoblotted as described above.

RESULTS

Pervanadate-induced hyperphosphorylation of RPTP α involves D2. The identification of substrates for several non-receptor PTPs has been done successfully in the past using catalytic site Cys to Ala or Ser mutations of PTPs (15–19). We used this approach to identify physiological substrates of RPTP α . RPTP α substrate trapping mutants were generated in which the active site Cys of one or both PTP domains was mutated to Ser (RPTP α -C433S, RPTP α -C723S, and RPTP α -C433SC723S). A hemagglutinin epitope tag (HA-tag) was cloned just downstream of the signal sequence in the extracellular part of RPTP α for efficient immunoprecipitation. SK-N-MC cells were transiently transfected with pSG-HA-RPTP α , C433S, C723S, C433SC723S, and treated with pervanadate for 30 min to increase P.Tyr levels of potential substrates, or left unstimulated. HA-RPTP α proteins were immunoprecipitated with 12CA5 and potential co-immunoprecipitating proteins were analyzed by immunoblotting, using anti-P.Tyr antibody (PY20) (Fig. 1, top panel). No obvious P.Tyr containing proteins co-immunoprecipitated with the substrate trapping mutant HA-RPTP α -C433SC723S. Surprisingly, pervanadate treatment of the cells induced high levels of Tyr phosphorylation of wild type RPTP α . Time courses of pervanadate treatment demonstrated that tyrosine phosphorylation of RPTP α occurred within 10 min and reached a plateau within 30 min (data not shown). In contrast, pervanadate induced only a very small increase in tyrosine phosphorylation of RPTP α -C723S and of RPTP α -C433SC723S (Fig. 1). Only a small, but significant decrease of tyrosine phosphorylation compared to wild type was observed for RPTP α -C433S

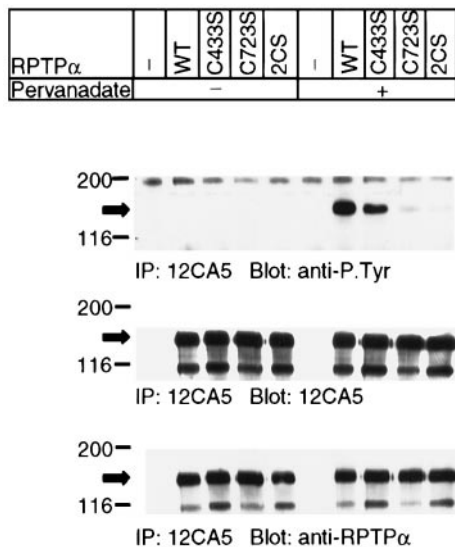


FIG. 1. D2 is involved in pervanadate-induced phosphorylation of RPTP α . SK-N-MC cells were transiently transfected with the expression vector pSG-5 (-), pSG-HA-RPTP α (WT), pSG-HA-RPTP α -C433S (C433S), pSG-HA-RPTP α -C723S (C723S), or pSG-HA-RPTP α -C433SC723S (2CS). The cells were treated with 1 mM orthovanadate and 1 mM H₂O₂ for 30 min, or were left unstimulated. The cells were lysed and HA-RPTP α proteins were immunoprecipitated with 12CA5 and analyzed by immunoblotting using anti-P.Tyr (PY20). The blot was stripped and reprobed with 12CA5 and anti-RPTP α antibodies, respectively. The molecular weights (in kDa) of marker proteins are indicated on the left. The position of RPTP α is indicated with an arrow.

(Fig. 1). The same results were obtained in stably transfected NIH 3T3 cells and transiently transfected COS-7 and HEK-293 cells, demonstrating that the observed effects were not cell type dependent (data not shown). These data show that RPTP α -D1 activity was not required for pervanadate-induced tyrosine phosphorylation, while D2 was somehow involved.

Pervanadate-induced tyrosine phosphorylation of RPTP α is not limited to Tyr789. Previously, we have demonstrated that RPTP α is phosphorylated on Tyr789 (20). In order to determine whether the effect of pervanadate treatment was limited to Tyr789, Tyr789 was mutated to Phe in wild type HA-RPTP α and C433SC723S (HA-RPTP α -Y789F and HA-RPTP α -C433SC723SY789F). SK-N-MC cells were transiently transfected with constructs as indicated and treated with pervanadate for 30 min or left untreated (Fig. 2). Pervanadate induced tyrosine phosphorylation of HA-RPTP α -Y789F to comparable levels as for wild type HA-RPTP α , indicating that pervanadate-induced tyrosine phosphorylation of RPTP α was not limited to Tyr789. The minor tyrosine phosphorylation of RPTP α -C433SC723S induced by pervanadate was completely abolished in the Y789F mutant background indicating that the minor phosphorylation of the Cys mutant is solely on Tyr789. Taken together, these data

indicate that the observed pervanadate-induced tyrosine phosphorylation of RPTP α was not limited to Tyr789.

Pervanadate-induced tyrosine phosphorylation of RPTP α is independent of RPTP α activity. One possible explanation why pervanadate induced tyrosine phosphorylation of wild type RPTP α , but not of the catalytically inactive mutant is that RPTP α might activate a kinase that in turn phosphorylates RPTP α . To test this hypothesis, SK-N-MC cells were transiently transfected with wild type HA-RPTP α or the HA-RPTP α -C433SC723S mutant in combination with untagged wild type RPTP α or the RPTP α -C433SC723S mutant and treated with pervanadate for 30 min. The HA-RPTP α proteins were immunoprecipitated with antibodies against the tag (12CA5) and the P.Tyr content was analyzed by immunoblotting using anti-P.Tyr antibodies (PY20) (Fig. 3, top panel). Pervanadate treatment induced tyrosine phosphorylation of wild type HA-RPTP α and this was not affected by co-expression of untagged wild type RPTP α or the RPTP α -C433SC723S mutant, indicating that RPTP α -C433SC723S does not exhibit dominant negative effects. In addition, tyrosine phosphorylation of HA-RPTP α -C433SC723S was not influenced by co-expression of untagged wild type RPTP α or the RPTP α -C433SC723S mutant. Apparently, the activity of wild type RPTP α did not induce hyperphosphorylation of RPTP α -C433SC723S. Taken together, it is un-

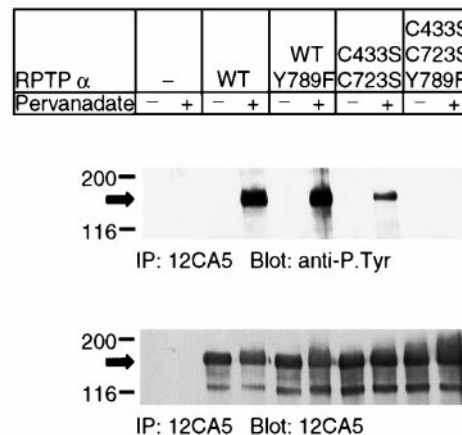


FIG. 2. Pervanadate-induced tyrosine phosphorylation of RPTP α is not limited to Tyr789. SK-N-MC cells were transiently transfected with 15 μ g pSG-5 (-), pSG-HA-RPTP α (WT), pSG-HA-RPTP α -Y789F (WT Y789F), pSG-HA-RPTP α -C433SC723S (C433S C723S), or pSG-HA-RPTP α -C433SC723SY789F (C433S C723S Y789F). The cells were treated with pervanadate for 30 min or left untreated, lysed and HA-RPTP α proteins were immunoprecipitated with 12CA5 and analyzed by immunoblotting using anti-P.Tyr (PY20). The blot was stripped and reprobed with MAb 12CA5. The molecular weights (in kDa) of marker proteins are indicated on the left. The position of RPTP α is indicated with an arrow.

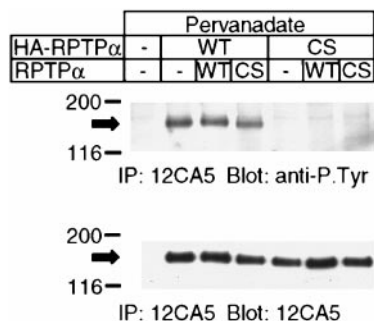


FIG. 3. RPTP α hyperphosphorylation is not an enzymatic effect. SK-N-MC cells were transiently transfected with 4 μ g pSG-5 (–), HA-tagged pSG-HA-RPTP α (WT) or pSG-HA-RPTP α -C433SC723S (CS) in combination with 12 μ g pSG-5 (–), untagged pSG-RPTP α (WT), or pSG-RPTP α -C433SC723S (CS) and treated with pervanadate for 30 min. The cells were lysed and HA-RPTP α proteins were immunoprecipitated with 12CA5 and analyzed by immunoblotting using anti-P.Tyr MAb (PY20). The blot was stripped and reprobed with MAb 12CA5. The molecular weights (in kDa) of marker proteins are indicated on the left. The position of RPTP α is indicated with an arrow.

likely that RPTP α activates a kinase that in turn phosphorylates RPTP α .

Another approach to test whether PTP activity is necessary for pervanadate-induced tyrosine phosphorylation is the use of other mutants that lack PTP activity. Mutation of Arg221 to Lys in PTP1B completely abolished PTP activity, rendering an inert protein (17). We mutated Arg in the signature motifs of RPTP α -D1 and D2 to Lys (R438K or R729K, respectively), which completely inactivated all PTP activity in these domains *in vitro* (data not shown). Despite the complete lack of activity, pervanadate still induced strong tyrosine phosphorylation of RPTP α -R438K and R729K, comparable to the levels seen for wild type RPTP α (data not shown). Taken together, these data suggest that RPTP α activity is not involved in pervanadate-induced tyrosine phosphorylation.

Pervanadate-induced tyrosine phosphorylation of RPTP α is located in the C-terminus. Finally, we mapped the pervanadate-induced tyrosine phosphorylation sites in RPTP α . To this end, SK-N-MC cells were transiently transfected with HA-RPTP α -Y789F and treated with pervanadate for 30 min. HA-RPTP α -Y789F mutant was used to exclude phosphorylation of Tyr789. Purified HA-RPTP α -Y789F protein was digested with various amounts of V8-protease and the P.Tyr content and identity of the fragments was analyzed by immunoblotting (Figs. 4A–4D), using anti-P.Tyr antibody (PY20), anti-HA-tag antibody (12CA5), an antibody against the C-terminus of RPTP α , or an antibody against the entire cytoplasmic domain of RPTP α (Fig. 4). The epitopes that are recognized by these antibodies are indicated in Fig. 4E. Treatment of RPTP α with increasing amounts of V8 protease re-

sulted only in a few major fragments containing P.Tyr. The largest fragment, a fragment of approximately 110 kDa, lacks the C-terminus, since this fragment was not recognized by the RPTP α antibody directed against the C-terminus (Fig. 4C), while the N-terminal HA-tag was present (Fig. 4B). Compared to the starting material, the 110 kDa fragment contained only very little P.Tyr, suggesting that the majority of the P.Tyr lies in the C-terminus. The 20 kDa fragment that was cleaved off of RPTP α to yield the 110 kDa fragment was rapidly degraded further, since it was not detected by the antibody against the RPTP α C-terminus (Fig. 4C) or the polyclonal RPTP α antibody (Fig. 4D). Three other major tyrosine phosphorylated fragments were observed: two fragments of approximately 64 kDa and a 30 kDa fragment. The two fragments of approximately 64 kDa were detected with PY20, the antibody against the RPTP α C-terminus, and the RPTP α polyclonal antibody (Fig. 4A, C, D), but not with 12CA5 (Fig. 4B) indicating that these fragments still contain the very C-terminal end but miss the N-terminus. The 30 kDa fragment lacks both C-terminus and N-terminus since it is only recognized by the RPTP α polyclonal antibody. Based on these data we conclude that pervanadate induced tyrosine phosphorylation throughout RPTP α , but mostly in the C-terminal 20 kDa of RPTP α . The observed tyrosine phosphorylation was not on Tyr789, since Tyr789 was mutated to Phe in Fig. 4. It is noteworthy that similar results were obtained when wild type RPTP α was used instead of the Y789F mutant. Mutation of the four most C-terminal tyrosine residues, Tyr772, Tyr776, Tyr782 and Tyr789, individually, had little or no effect on the pervanadate-induced tyrosine phosphorylation of RPTP α . However, mutation of all four C-terminal tyrosine residues reduced, but did not abolish pervanadate-induced tyrosine phosphorylation (data not shown). In conclusion, pervanadate predominantly induced tyrosine phosphorylation of the C-terminal 20 kDa in RPTP α on multiple sites.

DISCUSSION

Pervanadate treatment of cells induced strong tyrosine phosphorylation of RPTP α , which required intact RPTP α -D2, but not RPTP α -D1. Pervanadate-induced tyrosine phosphorylation of RPTP α was not restricted to Tyr789, and the kinase, responsible for pervanadate-induced tyrosine phosphorylation remains to be identified. Pervanadate-induced hyperphosphorylation localized predominantly to the C-terminus, although many other Tyr residues throughout RPTP α may be phosphorylated as well.

Inactivation of RPTP α by mutation by itself was not sufficient to induce tyrosine phosphorylation. RPTP α has autodephosphorylation activity, in that RPTP α dephosphorylated itself *in vitro*, and Tyr789 phosphorylation was enhanced in catalytically inactive RPTP α -

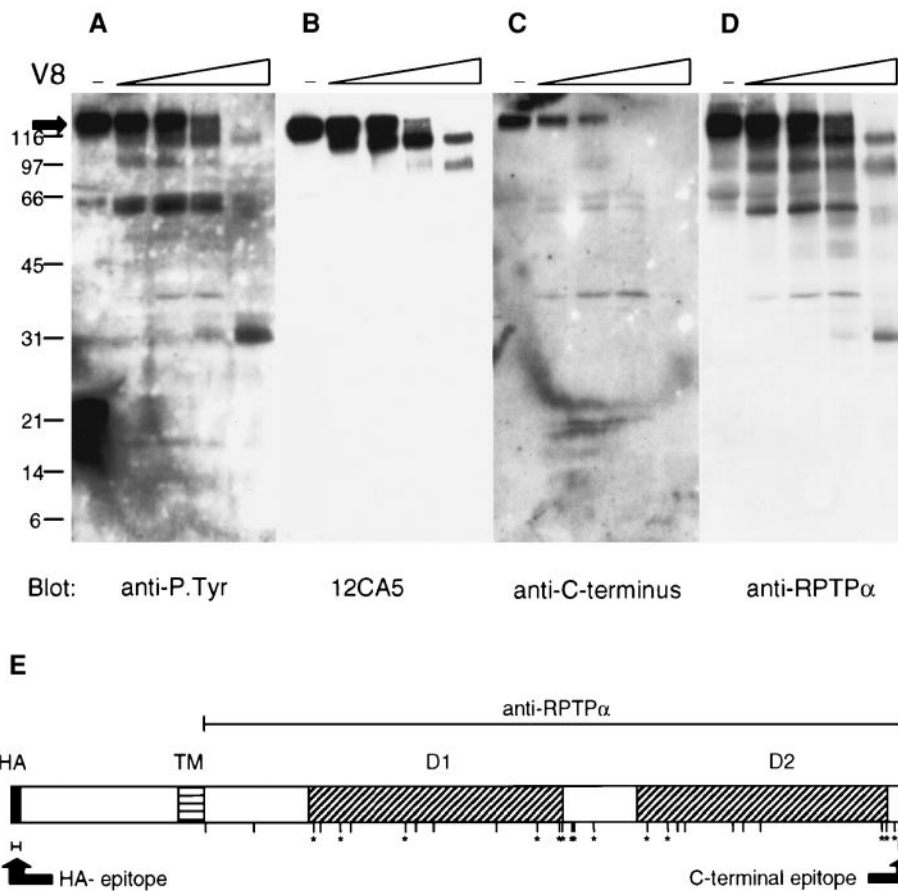


FIG. 4. Pervanadate-induced tyrosine phosphorylation of RPTP α predominantly in the C-terminal 20 kDa. SK-N-MC cells transiently transfected with 15 μ g pSG-HA-RPTP α -Y789F were incubated with 1 mM orthovanadate and 1 mM H₂O₂. The cells were lysed and HA-RPTP α proteins were immunoprecipitated with 12CA5. After electrophoresis on a polyacrylamide gel, RPTP α was cut out of the gel, eluted from the gel, precipitated with trichloroacetic acid, and resuspended in 50 μ l V8 digestion buffer with 0, 0.04, 0.1, 0.2, 0.5 units V8 protease and incubated at 37°C for 30 min. Samples were run on a 20% SDS polyacrylamide gel, and analyzed by immunoblotting as described under Experimental Procedures using sequentially: MAb PY20 (A), MAb 12CA5 (B), affinity-purified anti-RPTP α antiserum 5476, directed against the C-terminus (C), and affinity-purified anti-RPTP α antiserum 5478, directed against the entire cytoplasmic domain (D). The molecular weights (in kDa) of marker proteins are indicated on the left. The position of full length RPTP α is indicated with an arrow. A schematic representation of RPTP α is depicted in the lower panel (E). The HA-tag (HA), transmembrane domain (TM) and the two PTP domains (D1 and D2) are indicated. All tyrosines in the cytoplasmic domain are represented by a vertical line, and the ones that were mutated with an asterisk. The epitopes for 12CA5 (HA-epitope), the affinity-purified antibody 5476 directed against the C-terminus of RPTP α (C-terminal epitope), and the affinity-purified antibody 5478 directed against the full cytoplasmic domain of RPTP α (anti-RPTP α) are indicated.

C433AC723A (20). Since the PTP inhibitor pervanadate induced strong tyrosine phosphorylation of RPTP α , one might expect similar levels of tyrosine phosphorylation in catalytically inactive RPTP α in the case that RPTP α was solely responsible for its own dephosphorylation. Since RPTP α -C433S was clearly not highly phosphorylated, we conclude that RPTP α is not the major PTP responsible for keeping the basal tyrosine phosphorylation levels of RPTP α low.

A possible explanation why pervanadate induced tyrosine phosphorylation of wild type RPTP α , but not of RPTP α -C723S is that mutation of the catalytic site Cys may lead to conformational changes. Cys to Ser mutation is a single atom change, i.e., substitution of a sulfur for an oxygen. Therefore, it is unlikely that this

mutation will significantly alter the structure of the protein. Detailed analysis, using circular dichroism and ultraviolet-spectra, of structural differences between the wild type *Yersinia* PTP, YopH and the catalytic site Cys to Ser mutant demonstrate that the overall secondary and tertiary structural elements are similar for both proteins. However, conformational differences in the active site were found involving the position of the WPD loop harboring the Asp that functions as general base/general acid in catalysis (24). In contrast, no obvious differences were observed between the crystal structures of the wild type PTP domain and the catalytic site Cys to Ser mutant of PTP1B (25). The structure of RPTP α -D2 shows that there are no Tyr residues in or near the pocket of the active site (A.

Bilwes and J. Noel, personal communication), making it unlikely that conformational changes in the active site due to mutation of the active site Cys to Ser will lead to "masking" of Tyr residues that are no longer accessible for kinases in the Cys to Ser mutant. Yet, a small conformational change in RPTP α -D2 due to the Cys to Ser mutation may still be responsible for the differences in pervanadate-induced tyrosine phosphorylation (see below).

Another possibility why pervanadate induced tyrosine phosphorylation of wild-type RPTP α , but not of the mutant, might be that wild-type RPTP α activates a kinase that in turn phosphorylates RPTP α . This explanation, however, is unlikely since RPTP α -D2 contains very little activity, especially towards phosphopeptide substrates (11, 12). It has been suggested that RPTP α -D2 may have a high activity towards a specific (unknown) substrate. However, two residues that are highly conserved in RPTP-D1s (Tyr262 and Asp401 in RPTP α) and PTP domains of cytoplasmic PTPs, are essential for catalysis, and are not conserved in RPTP α -D2 and other D2s (26). Mutation of these two residues in RPTP α -D2 to the corresponding residues in D1 turned D2 into a very potent PTP domain (26, 27). In addition, pervanadate still induced hyperphosphorylation of RPTP α -R729K with a mutation that abolishes all PTP activity in D2 (data not shown). Taken together, these data suggest that RPTP α activity is not involved in pervanadate-induced tyrosine phosphorylation.

RPTP α is phosphorylated constitutively on Tyr789 (20). Here we demonstrate that mutation of Tyr789 to Phe has no effect on pervanadate-induced tyrosine phosphorylation (Fig. 2). Partial digestion of hyperphosphorylated RPTP α with V8 suggested that pervanadate-induced tyrosine phosphorylation was mainly localized in the C-terminus (Fig. 4). The fragment that contained the majority of the pervanadate-induced P.Tyr is a fragment of the C-terminus of approximately 20 kDa. In this fragment there are 7 tyrosines, 5 of which have the OH-group at the surface, based on the crystal structure of RPTP α -D2 (A. Bilwes and J. Noel, personal communication), making them potential targets for tyrosine phosphorylation. Pervanadate-induced tyrosine phosphorylation of RPTPs is not unprecedented, since CD45 became phosphorylated on tyrosine upon pervanadate treatment of T cells. Mapping of the tyrosine phosphorylation site led to the identification of Tyr1193, to the C-terminal side in CD45-D2 (28). The corresponding Tyr in RPTP α , Tyr772, is completely buried in the structure, inaccessible for kinases. Therefore, it is unlikely that pervanadate induces phosphorylation of this Tyr in RPTP α . In fact, mutation of Tyr772 to Phe had no effect on RPTP α hyperphosphorylation. The corresponding Tyr in other PTP domains, like Tyr 482 in

RPTP α -D1, is hidden in the structure as well (3). It will be interesting to see where Tyr1193 is located in the structure of CD45. We have mutated 15 out of the 27 Tyr residues in the cytoplasmic domain of RPTP α (Fig. 4E), but none of these single mutations had an effect on pervanadate-induced RPTP α tyrosine phosphorylation. Mutation of the four most C-terminal tyrosine residues reduced, but did not abolish pervanadate-induced P.Tyr levels, suggesting that pervanadate induced RPTP α phosphorylation on multiple sites.

We have shown that Src may be the kinase that phosphorylates RPTP α on Tyr789 (20). However, co-transfection of a constitutively active mutant of Src, SrcY529F together with RPTP α did not induce tyrosine phosphorylation of wild-type RPTP α or any of the mutants (data not shown) indicating that Src alone is not sufficient to induce hyperphosphorylation of RPTP α . The combination of co-transfection of SrcY529F and pervanadate treatment induced tyrosine phosphorylation of wild-type RPTP α , but not of the catalytic site Cys mutants, similar to pervanadate treatment alone (data not shown). In addition, co-transfection of inactive Src, SrcK297M, which may have dominant negative activity did not significantly lower pervanadate-induced tyrosine phosphorylation levels of RPTP α (data not shown). Therefore, the mechanism of pervanadate-induced tyrosine phosphorylation of wild-type RPTP α probably does not involve Src or any downstream targets of Src.

Several reports suggested that D2s may be involved in protein-protein interactions (6, 29, 30). The most likely explanation for the effect of the Cys to Ser mutation in RPTP α -D2 on pervanadate-induced tyrosine phosphorylation is that this mutation induced a small conformational change in the active site like YopH (24), thereby abolishing binding to a kinase, or a protein complex containing a kinase, that phosphorylated RPTP α on tyrosine. Presumably, this interaction was weak, since we have not been able to detect PTK activity co-immunoprecipitating with RPTP α . Moreover, yeast genetic two-hybrid screens, using RPTP α -D2 as a bait, did not lead to identification of any PTKs as interacting (prey) proteins (C.B., A.B., and J.d.H., unpublished results).

In conclusion, a constitutively active kinase phosphorylated RPTP α on tyrosine following treatment of cells with the non-selective PTP inhibitor pervanadate. Mutation of the catalytic site Cys to Ser in D2 abolished pervanadate-induced RPTP α tyrosine phosphorylation, demonstrating that RPTP α -D2 was somehow involved. It will be interesting to identify the kinase responsible for pervanadate-induced tyrosine phosphorylation of RPTP α and test whether the Cys to Ser mutation in RPTP α -D2 blocks the interaction with this kinase.

ACKNOWLEDGMENTS

We thank Alex Bilwes and Joe Noel (Structural Biology Laboratory, The Salk Institute, La Jolla, CA) for providing the coordinates of the crystal structure of RPTP α -D2 prior to publication. In addition, we thank John Overvoorde for technical assistance. This work was supported by the Research Council for Earth and Life Sciences (ALW) with financial aid from the Netherlands Organization for Scientific Research (NWO) (to A.B.), and a grant from the Dutch Cancer Society (to J.d.H.).

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